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13. ABSTRACT (Maximum 200 Words)

The main purpose of this Career Development Award is for the P.I. to receive training in the field of Cancer Biology and Virology, so that she can apply her expertise, Magnetic Resonance Imaging, for developing non-invasive techniques to monitor gene therapy induced cellular and vascular changes in breast cancer. In Yr-01 I have received some training and attended several courses. In Yr-02 I have started to expand the experiments by using gene therapy delivered with the adenovirus system to treat animal cancer models, and performing MRI studies to measure the volumetric and vascular changes. A research assistant has been recruited and trained in assisting me performing all aspects of the study, including carrying out the experiments and data analysis. I have also spent some effort in doing extensive literature search to study the recent developments in the application of angiogenesis or anti-angiogenesis therapy using gene delivery systems. The search results have been used in a proposal submitted to NIH, and have successfully obtained funding. In Yr-03 we will continue to finish the specific aims proposed in the original proposal, with the main effort focusing on the correlation between the MRI measurements of cellular and vascular changes with the underlying biological changes occurring in the tissue, e.g. the change of vessel density.

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FOREWORD

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(4)   **Table of Contents**

(5)   Introduction

This training proposal (Career Development Award) has two major goals: 1) Personally for me to go through the necessary training and become an independent breast cancer investigator, and 2) Scientifically to find the non-invasively measurable imaging parameters that reflect the underlying biological changes to predict the eventual efficacy of gene therapy. As a physicist working in the field of medical imaging research for years, I have accumulated sufficient experience in the imaging technology, especially for the application in cancer diagnosis and prognosis. But my training and experience in tumor biology and gene therapy is not adequate. Therefore, to carry out the study, I need to receive training in the application of recombinant virus technology for gene therapy. The training will catalyze my development in the promising field of gene therapy and upgrade my status to an independent investigator.

After gaining sufficient experience, I will choose three appropriate recombinant viral systems to work with. Ideally the three viral systems should work through the mechanisms of direct cell killing, indirect killing via microvasculature damage, and a combination of both, respectively, in achieving the therapeutic effect. MR imaging and biological tissue examination techniques will be combined to study the prognosis of tumors undergoing these different types of gene therapy. The longitudinal structural and vascular changes in tumors will be measured with MR imaging. Also, the associated biological or pathological characteristics of the tumors before and at certain times after the treatment will be measured. The results obtained from the imaging study will be correlated with the underlying biology to investigate their relationships. By working with these different mechanisms, I hope to study the limit and the extent of the information that MRI measurements of cellular volume and vascular changes can provide.

(6) Body

In the proposal I planned to achieve 6 specific aims during the three year funding period:
**   1. Obtain basic training in general tumor biology and standard laboratory work,
**   2. Pursue specific training in the application of recombinant virus technology for gene therapy,
*   3. Choose and, if necessary, develop three appropriate recombinant viral systems that are expected to cause direct cell killing, indirect killing via microvasculature damage, and a combination of both, respectively,
*   4. Study the longitudinal structural and vascular changes in tumors receiving the three viruses with MRI,
*   5. Study the associated changes in the biological or pathological characteristics of the tumors at various times after the treatment,
6. Correlate the changes in the MRI parameters with the underlying biological changes to establish the relationships between them.
"**" completed in Yr-01, "*" on-going projects in Yr-02

I have been working on Aims 3-5 during the Yr-02 of the project period. For Aim 3 I have been working on searching the appropriate systems for the proposed study, i.e. to find genes that can be encoded into AD to reach therapeutic effect by direct cell killing and inhibition of angiogenesis. I have done extensive literature search, and have included the results in the proposal submitted to NIH. The search results have been summarized in Yr-01 report, not repeated here. I have also obtained a plasmid containing the VEGF 121 gene (Vascular Endothelial Growth Factor), from Dr. A. Harris at the University of Oxford, UK. Initially we were planning to transfect the gene into adenovirus. However, this requires a great effort. The staff at the Vector Center at our institute left the job, and currently there is no one available to carry out this difficult task. It also requires a great resource, which is not supported by this training grant.

Therefore, instead of spending effort on developing this vector, we will focus on using imaging techniques to monitor the changes taking place in cancer after infected with adenovirus carrying different cytokines. The genes which had been studied included mouse interferon γ (IFN-γ), human transforming growth factor β (TGF-β), and mouse interleukin 1-α (IL1-α), or the marker gene β-gal. We will focus on the correlation between the results obtained from in –vivo imaging and the underlying changes measured by immunohistochemical analysis. My collaborator at Oncotech Inc has developed the techniques to stain the vessels from rat tissue. In this report I will describe the results that we have obtained from the imaging experiments. The immunohistochemical tissue examination will be performed in Yr-03, and the results will be correlated with imaging findings.

Next I will describe the summarized methods for carrying out the gene therapy monitoring study proposed in this application. The initial results have been reported in Yr-01 report, also published as a journal paper. I will describe the results from an expanded study in this section.

Methods:

Tumor Model:

C6 glioma cells implanted subcutaneously and bilaterally into the rear haunch of 5 Wistar rats (5 million cells were injected). Two weeks after the injection the tumors reached to 0.7 cm in diameter. The baseline MRI study was performed.

Preparation of Recombinant Adenovirus:

A. Purification of Plasmid DNA
B. DNA Transfections for Rescue of Recombinant
   Adenovirus Vectors: Using Lipofectamine methods
C. Amplification of a Clonal Viral Stock
D. Extraction of Viral DNA from 293 cells
E. Titration, Large Scale Propagation and Purification of Adenovirus

Adenovirus encoding:
mouse interferon gamma (IFN-γ)
mouse interleukin 1 alpha (IL 1-α)
human transforming growth factor beta (TGF-β)
marker gene (β-gal)

Gene Expression of C6 glioma cells to the virus system:
The in-vitro and in-vivo expression of β-galactosidase were confirmed.
In-vitro experiments showed that corresponding to the MOI (multiplicity of infection) of 10, 1, and 0.1, 80%, 50%, and 5% of the cultured cells expressed the recombinant b-gal gene. The vivo experiments showed that the recombinant gene expression was localized at the site of viral injection.

Treatment:

One day after the baseline MRI study, three animals received left-side intratumoral injection of 2x108 plaque forming units of virus, two rats received saline as control.

MRI Studies:

1. Interleaved T2-weighted images (fast SE) across the tumor for measurement of the total tumor volume.
2. Select a slice through the center of tumor for dynamic study. Also select a slice through the liver to measure the liver kinetics.
3. Inject 0.1 mmol/kg DTPA after 4 pre-contrast images had been acquired.
4. The MRI studies were repeated on day 4, 7, 11, and 15 after the baseline study (day 0).

Results of Expanded Studies:

The study protocol was applied to a large group of rats (n=21) with bilateral tumors. One side of tumor was treated with adenovirus carrying IL-1α, IFN-γ, or β-gal gene. The longitudinal volumetric growth rates and the vascular parameters were measured. According to their growth pattern, the tumors were categorized into three groups:

Group1 (Grow-Grow):   grow at day 4 and further grow at day7
Group2 (Grow-Reg) :   grow at day 4 and regress at day 7
Group3 (Reg-Reg)   :   regress at day 4 and further regress at day 7

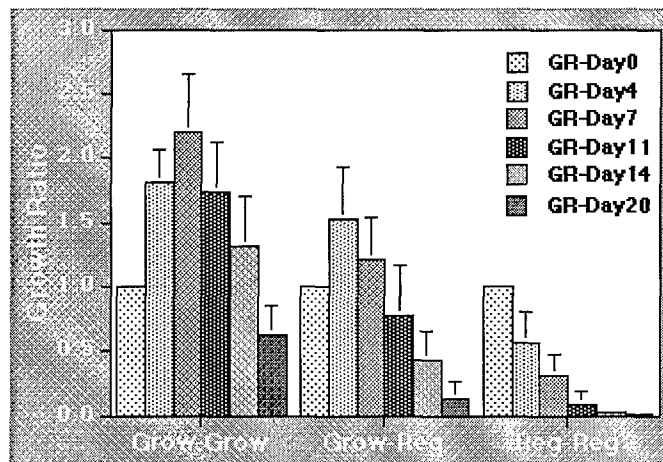The longitudinal growth ratio in each of the 3 groups are plotted in Figure 1.

Figure 1: The mean growth ratio (the volume of tumor at follow-up studies compared to the baseline volume) of tumors in the three groups. The vascular properties were measured to investigate whether they can be used to differentiate these 3 groups.

The table below summarizes the distribution of all 42 tumor (treated side or contralateral side) with the three virus systems into these three groups. The treatment did not cause consistent modulation of tumor growth.

Distribution of tumors in 3 groups of various growth pattern

21 rats 42 tumors	IL-1α (n=8)		IFN-γ (n=5)		β-gal (n=8)	
	treat	contra	treat	contra	treat	contra
Grow-Grow (n=12)	3/8	4/8	0/5	1/5	2/8	2/8
Grow-Reg (n=13)	2/8	1/8	1/5	3/5	4/8	2/8
Reg-Reg (n=17)	3/8	3/8	4/5	1/5	2/8	4/8

We were interested in studying whether the baseline vascular volume or the changes of vascular volume can predict their future growth pattern as demonstrated in the pilot study. Figure

2 a-d show the enhancement kinetics measured from the tumors in each group. In group 1 the tumors are further separated into continuous growth after day 11 or regression after day 11 (shown in Fig.2a and Fig.2b). However, none of these vascular parameters could significantly differentiate the tumors in these 3 groups. One reason could be that the contrast agents used for this study is an extracellular agent, which could not be used to probe the vascular properties as well as the blood pool agents.

Summary of the pilot Study from 5 rats (10 tumors):

1. The changes in vascular volume, as analyzed from the kinetics of Gd-DTPA measured with dynamic MRI, is predictive of future tumor growth.

2. In all 6 tumors which were increasing in size at day 4, a decrease in tumor size several days later was preceded by a decrease in vascular volume at that time.

3. We hypothesize the therapy induced a modulation in the immune response of the host animal to the tumor. rTGF-β and rIFN-γ resulted in an accelerated tumor rejection. rIL1-α led to a delayed (2 weeks) onset of tumor rejection.

Summary of the extended study from 21 rats (42 tumors):

1. The gene therapy treatment on this tumor model modulated the natural growth pattern. Some kept growing, some grew bigger then regressed, and some exhibited continuous regression.

2. As more subjects were studied, the initial results that vascularity change predicted future volumetric growth did not hold on. However, the MRI system is expected to be more useful for monitoring the effect of anti-angiogenic therapy.
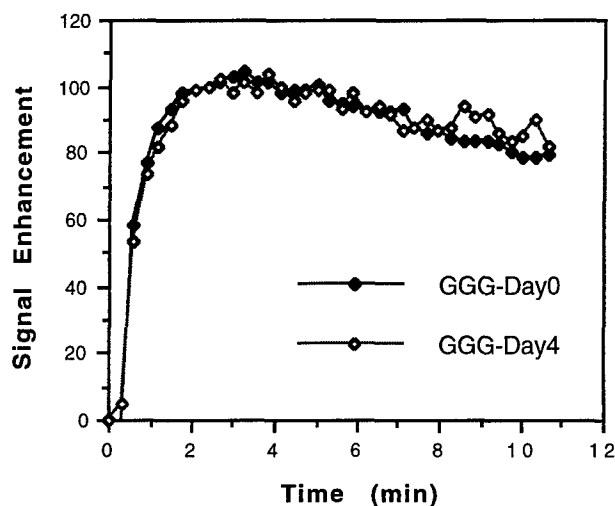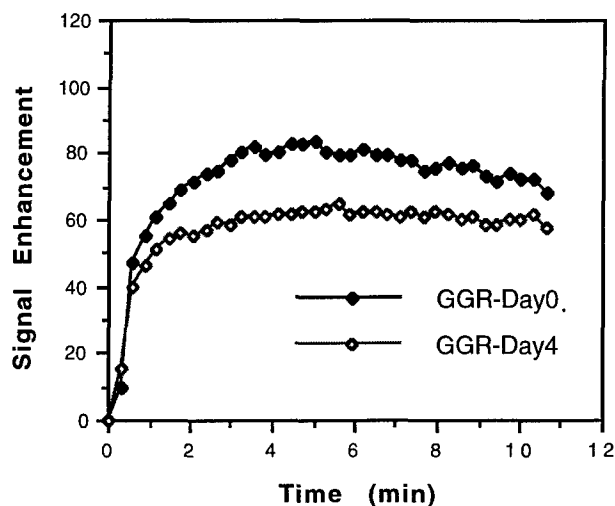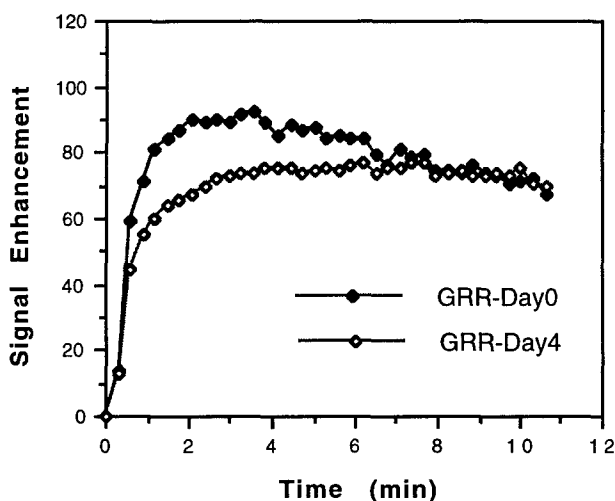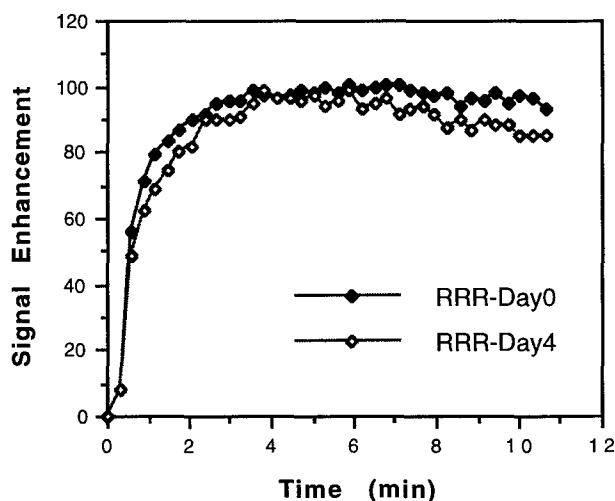
Fig. 2a

Fig. 2b

Fig. 2c

Fig. 2d

Figure 2: The enhancement kinetics of the 4 groups of tumors, stratified by their different growth patterns. GGG: grow at Day-4, Day-7, Day-11, GGR: grow at Day-4, Day-7, regress at Day-11; GRR: grow at Day-4, regress at Day-7, Day-11, RRR: regress at Day-4, Day-7, Day-11. Comapring the enhancement kinetics at day 7 in the GGG group to that in the GGR group, the sustained enhanceemt in the GGG group predicted the future growth at Day-11, whereas the reduced enhancement in the GGR group predicted the regression at Day-11. However, this observation does not hold up in the GRR and RRR group.

(7) Appendix

1) A bulleted list of key research accomplishments:

- Trained a research assistant for cell culture, animal work, and tissue preparation techniques to participate in this project.
- Continued to work on the experiments using magnetic resonance imaging to monitor the gene therapy induced changes.
- Worked with the researchers at Oncotech Inc. for developing the immunohistochemical methods to stain the vessels from rat tissue.
- Developed the methods to attach MR contrast agent, Gadolinium, to the adenovirus, so that the fate and distribution of adenovirus in the host body and the cancer can be monitored using non-invasive imaging techniques.

2) A list of reportable outcomes

- One paper entitled "Prediction of Gene Therapy Induced Tumor Size Changes by the Vascularity Changes Measured Using Dynamic Contrast Enhanced MRI" has been published by "Magnetic Resonance Imaging", enclosed herein.
- Gave a presentation at the Chao Family Comprehensive Cancer Center Annual meeting.
- Attended the 2000 DOD Era of Hope meeting to give a platform talk and present a poster.
- Submitted a proposal entitled " Synthesis of a Detectable Neovasculature Targeted Adenovirus" to NIH, revised it twice and have successfully obtained the funding.

3) A copy of each of the above cited manuscript and abstract

Two Abstracts:

Lydia Su; Monitoring Gene Therapy Induced Responses in Cancer by MRI. in Proceedings, Chao Family Comprehensive Cancer Center Conference, Palm Springs, October, 1999

M.-Y. Su, J. A. Taylor, L. P. Villarreal and O. Nalcioglu; Prediction of Gene Therapy-Induced Tumor Size Changes by the Vascularity Changes Measured Using Dynamic MRI. in Proceedings, Era of Hope Department of Defense Breast Cancer Research Program, Atlanta, 2000. Page 245.

One Journal Paper:

M.-Y. Su, J. A. Taylor, L. P. Villarreal and O. Nalcioglu; Prediction of Gene Therapy-Induced Tumor Size Changes by the Vascularity Changes Measured Using Dynamic Contrast Enhanced MRI. Magnetic Resonance Imaging 18 (2000) 311-317.

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ABSTRACT FORM

As new advances in immunotherapy in cancer treatment emerge, there is an ever increasing need to develop treatment monitoring systems to determine the efficacy of therapy as early as possible for optimization of treatment regimens. In this study we investigated the feasibility of using the non-invasive MRI to predict treatment efficacy prior to changes in tumor growth.

A C6 glioma model was treated with three recombinant adenoviruses expressing mouse interferon γ (IFN-γ), human transforming growth factor β (TGF-β), and mouse interleukin 1-α (IL1-α). The pre-treatment baseline MRI study was conducted. The MRI protocol included a T2-w sequence for volumetric measurements, and a dynamic T1-w sequence to measure the kinetics of Gd-DTPA for vascular measurements. Within 24 hours after the baseline MRI, 3 animals received left-sided intratumoral injection of 2×10^8 plaque forming units of virus, one from each kind. Two animals received saline as controls. The post-treatment experiments were repeated at 4, 7, 11, and 15 days after the baseline studies.

The longitudinal volumetric and vascular changes were measured. All 4 control tumors showed the same growth pattern. They continued to grow between baseline and day 4, then started to regress at day 7. IL1-α treated tumor continued to grow larger till day 15, while TGF-β and IFN-γ treated tumors had regressed by day 4. The vascular volume changes of the control and IL1-α treated tumors at day 4 were predicted of their volumetric changes at day 7.

The outcome of the study suggested that the self-immune responses of the animals for tumor rejection were perturbed by the treatment (IFN-γ and TGF-β accelerated the rejection while IL1-α delayed it). We demonstrated that the changes of vascular volume, determined using dynamic contrast-enhanced MRI, is predictive of future tumor growth.

Abstract Title:	Monitoring Gene Therapy Induced Responses in Cancer by MRI
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PREDICTION OF GENE THERAPY INDUCED TUMOR SIZE CHANGES BY THE VASCULARITY CHANGES MEASURED USING DYNAMIC MRI

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As new advances in immunotherapy in cancer treatment emerge, there is an ever increasing need to develop treatment monitoring systems to determine the efficacy of therapy as early as possible for optimization of treatment regimens. We studied the changes of tumor size after gene therapy treatment and its relationship with the changes of vascular volume as measured by using dynamic contrast enhanced MRI, to investigate the feasibility of using the non-invasive MRI to predict treatment efficacy prior to changes in tumor growth.

The study was carried out using a spontaneously regressing rat tumor model (C6 Glioma grown subcutaneously in rats). The tumor model was treated with three recombinant adenoviruses expressing mouse interferon γ (IFN-γ), human transforming growth factor β (TGF-β), and mouse interleukin 1-α (IL1-α). The in-vitro and in-vivo gene expression was first tested using recombinant human adenovirus containing the β-galactosidase gene driven with a CMV promoter. When the tumor reached 0.7 cm in diameter, the pre-treatment baseline MRI study was conducted. The MRI protocol included a T2-weighted pulse sequence for volumetric measurements, and a dynamic T1-weighted pulse sequence to measure the kinetics of Gd-DTPA for vascular measurements. Within 24 hours after the baseline MRI, 3 animals received left-sided intratumoral injection of 2×10^8 plaque forming units of virus, one from each kind. Two animals received saline as controls. The post-treatment experiments were repeated at 4, 7, 11, and 15 days after the baseline studies.

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Prediction of gene therapy-induced tumor size changes by the vascularity changes measured using dynamic contrast-enhanced MRI☆

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Abstract

We studied the changes of tumor size after gene therapy treatment and its relationship with the changes of vascular volume as measured by dynamic contrast-enhanced magnetic resonance imaging (MRI), to investigate whether the vascular changes is predictive of tumor regression. The study was carried out using a spontaneously regressing rat tumor model (C6 Glioma grown subcutaneously in rats). Three rats were treated with recombinant adenoviruses expressing three genes, mouse interleukin 1-α (IL1-α), mouse interferon γ (IFN-γ), and human transforming growth factor β (TGF-β), one from each kind. Two rats were treated with saline as controls. Longitudinal studies were performed to monitor the changes of tumor volume (based on T_2-weighted images) and the vascular volume (based on dynamic contrast enhanced images). In untreated animals, tumor regression was preceded by several days with a decrease in vascular volume. When the tumor growth was perturbed by expression of mouse IL-1α, the increase in vascular volume was correlated with the continuing growth in size, and the decrease in vascular volume was predictive of the onset of tumor regression. As new advances in immunotherapy in cancer treatment emerge, the ability to determine the efficacy of therapy as early as possible will enable optimization of treatment regiments. The vascularity changes measured by dynamic MRI may provide a means to serve for this purpose. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Gene therapy; Cancer therapy; Vascularity

1. Introduction

As new advances in immunotherapy in cancer treatment emerge, there is an ever-increasing need to determine the efficacy of therapy as early as possible in order to optimize treatment regimens. Unfortunately, the conventional imaging techniques determine tumor size but do not predict future tumor size changes. In the last few years, there has been increased evidence of the role of angiogenesis and vascularity in the growth of cancer and its response to therapy [1,2]. However, the prevailing methods assessing the level of angiogenesis, which involve analyzing tumor biopsies, are both invasive and time consuming and thus not

very practical. It would be extremely useful to develop a non-invasive technique to predict treatment efficacy prior to changes in tumor growth.

In the current study, we used an allogeneic rat tumor model and treated the tumors with adenoviruses expressing three various genes: mouse interferon gamma (IFN-γ), mouse interleukin 1 alpha (IL1-α) and human transforming growth factor-β (TGF-β), and monitored their volumetric changes after the treatment. T_2-weighted images were used to measure the total volume of tumors, utilizing the high T_2 relaxation times of tumors (associated with high water content). Dynamic contrast-enhanced magnetic resonance (MR) imaging is a non-invasive technique that can be used to measure the time-varying distribution of contrast agents within tissues, from which the information of vascular volume and permeability can be derived [3]. There is a strong evidence that vascular volume and permeability are two important properties in diagnosis and prognosis of a tumor. For instance, these parameters can be used in differential

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diagnosis between benign and malignant tumors and in determining the aggressiveness of tumor growth [4–6]. It has also been shown that these parameters are sensitive to chemotherapy and radiation therapy and thus may be used to aid in therapy planning or even predict the treatment outcome [7–11].

In the dynamic contrast-enhanced study, we used the clinically available small molecular agent, Gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA, with a molecular weight of 0.57 kD). By monitoring the signal intensities in the T_1-weighted images acquired dynamically after injection of contrast agents, the kinetics of the agent within a tumor can be measured, which can then be analyzed with a pharmacokinetic model to derive the vascularity information. We demonstrated that vascular volume changes, measured by dynamic contrast-enhanced MRI, correlated with future changes in tumor growth in the controls and gene therapy treated tumors.

2. Materials and methods

2.1. Establishment of an allogeneic bilateral rat tumor model

All procedures were in accordance with protocols approved by the University Institutional Animal Care and Use Committee. Wistar rats (obtained from Charles River, Wilmington, MA) were injected with 5×10^6 C6 cells bilaterally and subcutaneously into the rear haunch of each rat. C6 cells are a N-nitrosomethylurea-induced glial tumor line derived from BDIX mice [12], and were obtained from American Type Culture Collection (Manassas, VA). In our laboratory, tumors grew in Wistar rats for 3 weeks before they slowly spontaneously regressed.

2.2. Preparation of recombinant adenoviruses

The construction and propagation of replication incompetent human recombinant adenovirus 5, containing the exogenous genes, driven by the human cytomegalovirus immediate early promoter, were previously described [13]. The cDNA for the genes encoding for mouse interleukin 1α, mouse interferon γ, and human transforming growth factor-β, were obtained from ATCC and cloned into the polylinker of the plasmid containing the left LTR of adenovirus (pLAD) and subsequently cotransfected with PJM17 into 293 cells to obtain recombinant virus plaques.

2.3. Test of in vitro and in vivo gene expression using beta-gal as markers

The in vitro gene expression was tested using recombinant human adenovirus containing the β-galactosidase gene driven with a CMV promoter (rβ-gal). The cells were cultured in 6-well plates to 60–80% confluency. At this time media was removed and 200 μL of media containing recombinant rβ-gal virus [multiplicity of infection (MOI); 10, 1, 0.1] was overlaid onto each plate and incubated for 1 h. Media were subsequently replaced, and the cells were incubated for 37 additional hours. The cells were then washed, fixed and stained using X-gal, a substrate for β-galactosidase gene product. Using this method, 80%, 50%, and 5% (corresponding to 10, 1, and 0.1 MOI, respectively) of the cultured cells were shown to express the recombinant gene.

The in vivo animal study was performed using a similar assay for gene expression. rβ-gal was injected intratumorally into pre-established tumors. After 36, 72, and 98 h, the tumors were excised, fixed, sectioned, and stained with X-gal. The results showed that recombinant gene expression was localized at the site of viral injection.

2.4. MRI measurement procedures

The baseline study was conducted when the size of tumor reached 0.7 cm in the longest dimension, approximately 2 weeks after tumor cell inoculation. Rats were anaesthetized with an intramuscular injection of Ketamine (87 mg/kg) and Rompun (13 mg/kg). They were then immobilized on a board to prevent incidental movement. An intravenous line via tail vein was established, using a 25-gauge butterfly syringe (Becton Dickinson, Sandy, UT), prior to imaging. All experiments were performed on a 1.5 Tesla General Electric Signa Scanner using a GE linear head coil. Once positioned in the scanner, alignment markings were placed on the center of the bilateral tumors in each animal to ensure the same positioning in the subsequent follow-up studies. A set of T_2-weighted images (3-mm thick each) covering the whole tumor were acquired to measure the tumor volume, using a fast-spin echo pulse sequence with [TR = 3s, TE = 112 ms, echo train = 8, matrix size = 256 × 256, field-of-view (FOV) = 16 cm]. Two interleaved scans were performed to collect the odd number and even number slices separately to obtain good quality images from the whole tumor without gaps. Then a slice (5-mm thick) through the center of the tumor was prescribed for the dynamic T_1-weighted MRI study. Another T_2-weighted image of this 5-mm thick slice was acquired to serve as the reference image for the location of the tumor, since it clearly demonstrated the margin of the tumor. T_1-weighted dynamic contrast-enhanced imaging was performed using the SE pulse sequence with a TR/TE = 100/14 ms with a temporal resolution for each image of 14 s. The FOV = 16 cm and matrix size = 256 × 128. The contrast agent, Gd-DTPA (0.1 mmol/kg, Magnevist®, Wayne, NJ), was injected via the tail vein after acquiring 4 pre-contrast T_1-weighted images. The sequence was applied to continuously acquire the post enhanced images for 14 min after injection.

2.5. Therapy administration and longitudinal follow-up

The treatment was given within 24 h after completion of the baseline MRI study. In three rats, the right side of the bilateral tumor was injected with adenoviruses expressing, mouse interleukin 1-α (IL1-α), mouse interferon γ (IFN-γ), and human transforming growth factor β (TGF-β), one from each kind. Animals received left-sided intratumoral injection of 2×10^8 plaque forming units of virus. Intratumoral injection was used to enhance the replication efficiency of the genes. Two rats were treated with saline as controls. The baseline study was noted as day 0. On days 4, 7, 11, and 15, the follow-up studies were conducted. The T_2-weighted images across the whole tumor were taken for measurements of the total tumor volume. In the follow-up studies, the dynamic contrast enhanced study was conducted when the tumor size was larger than 5 mm in the axial dimension. The tumor volume and the vascular volume at each measurement time point were analyzed using the procedures described in the next section.

2.6. MRI image analysis procedures

For each tumor slice, a region of interest (ROI) was manually outlined from the T_2-weighted images acquired across the whole tumor. The total tumor volume was calculated by summing over all outlined regions in the set of T_2-weighted images. Then, based on the anatomic image of the tumor slice selected for dynamic contrast enhancement study, an ROI was outlined. The mean signal intensity of this tumor ROI in each pre- and post-contrast T_1-weighted image was measured. The enhancement was calculated by subtracting the pre-contrast signal intensity (averaged over the four pre-contrast images) from the post-contrast signal intensity at every time point. From our previous experience, the enhancement level is in the linearity range with concentration; therefore, it is approximately proportional to the concentration of the contrast agent. The time course of the enhancement represents the kinetics of Gd-DTPA concentration in the tumor.

The kinetics from the liver was also measured to serve as a reference. The liver is composed of discontinuous capillaries; the contrast agents can easily diffuse in and out of the vessels and reach equilibrium between these two compartments. Because the equilibrium can be reached quickly, the agent is being distributed in the whole extracellular space in the liver and that the measured kinetics approximates the vascular kinetics of the contrast agent. We have previously developed a pharmacokinetic analysis that models the distribution of the contrast agents between intravascular and extravascular compartments [14]. After vascular and interstitial contributions are separated, the derived parameters can be used to quantitatively characterize the vascular volume and permeability. The vascular component represents signal generated from within the blood vessels, while the extravascular component is generated from signal derived from the extravascular space. By referencing to the extracellular volume of liver (24%), the separated vascular kinetics can be converted into the fractional vascular volume. The details of the procedure have been described in a previous publication [6]. The analysis in the follow-up studies followed the same procedure. From the ratios of the tumor sizes or vascular volumes taken on separate days longitudinally, relative changes in the sizes or vascular volumes of the tumors were determined for comparison.

Statistical analysis was performed using student's t-test, with a significance level of 0.05.

3. Results

We used a subcutaneous bilateral allogeneic rat tumor model to evaluate longitudinal tumor size and vascular changes using MRI. We took advantage of this allogeneic model and the alterations in their growth patterns after perturbed by gene therapy to evaluate the effectiveness of MRI assessment of tumor growth and regression. The relationships between the changes of tumor size and the vascularity were compared to investigate whether the tumor regression can be predicted by the vascular changes.

3.1. Changes in tumor volumes

Figure 1 shows the T_2-weighted images taken from the central cut of the bilateral tumors from all five rats during the longitudinal study period. The images shown are from a 5-mm slice, the same slice taken for the dynamic study. The bilateral tumors are clearly demonstrated as bright regions on the T_2-weighted images. The top two rats are the controls. All four tumors show the same pattern. They grew larger at day 4, and started to decrease at day 7. By day 15, they were almost gone. In contrast, note that the IL1-α-treated tumor grew larger at day 4 and even larger at days 7 and 11, then started to regress at day 15. On the other hand, the TGF-β-treated tumor started to regress at day 4. The IFN-γ-treated tumor almost disappeared at day 4. Although only the left tumor received intratumoral treatment, the effects were bilateral, i.e., both tumors showed the same growth pattern.

T_2-weighted consecutive thin slices (3-mm thickness) were acquired across the whole tumor to determine the tumor volumes. Volumetric measurements were taken at days 0 (baseline), 4, 7, 11, and 15. Since the bilateral tumors exhibited the same growth pattern, they were categorized as one group. All four control tumors were categorized as one control group. The relative changes of the size of tumors in each study group at day 4 compared to day 0, and at day 7 compared to day 4 are listed in Table 1, and the changes at day 11 compared to day 7 are listed in Table 2. The tumors in the control rats (rat A–B) continued to grow between baseline and day 4, increasing in size by an average of 73%. The recombinant mouse interleukin 1 alpha (rIL-1α) inoc-
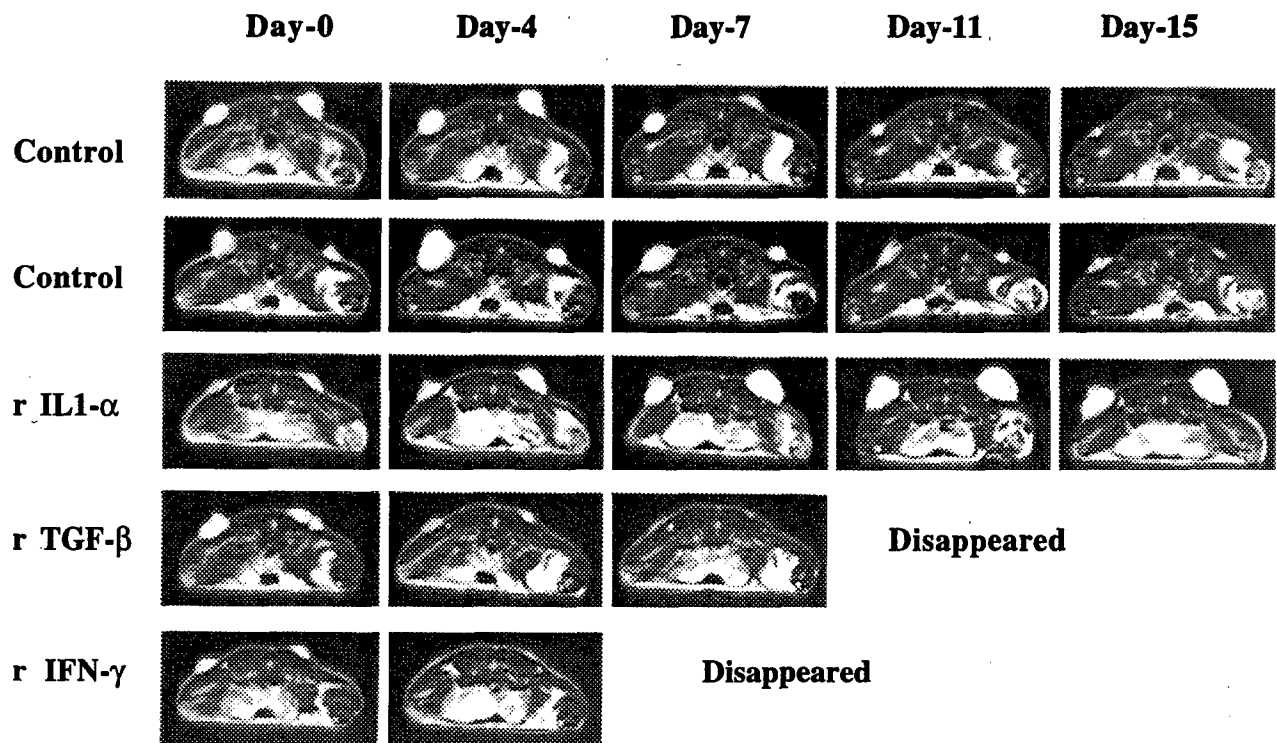
Day-0 **Day-4** **Day-7** **Day-11** **Day-15**

Fig. 1. The T_2-weighted MR images of Wistar rats bearing subcutaneous bilateral C6 tumors. The animals were in prone position, and the image was taken from an axial cut (5-mm thickness) through the center of both tumors. Infected animals received 2×10^8 plaque forming units (PFU) of recombinant human adenovirus, containing cytokines as indicated, via an intratumoral injection into the left tumor one day after baseline measurements. Rat A–B were non-treated, Rat C was inoculated with rIL-1α, Rat D was inoculated with rTGF-β, and Rat E was inoculated with rIFN-γ. The control tumors started to regress at day 7, and totally disappeared by day 15. rTGF-β and rIFN-γ resulted in an accelerated tumor rejection, while rIL1-α led to a delayed onset of tumor rejection.

ulated rat (rat C) continued to grow over the same period, increasing in size by an average of 140%. However, the tumors inoculated with recombinant human transforming growth factor beta (rTGF-β) (rat D) and with recombinant mouse interferon gamma (rIFN-γ) (rat E) rats had started to regress by day 4. By day 7, control tumors began to regress, showing an average decrease of 40%, and the rTGF-β and rIFN-γ infected tumors disappeared, while the tumors in the rIL-1α infected mouse continued to grow by an average of 159%. By day 11, the control tumors became much smaller, showing an average decrease of 80% compared to day 7. The rIL1-α treated rats had a much slower growth rate, from

159% increase at day 7 down to 40% increase at day 11. At day 15, the control tumor almost disappeared, and the rIL1-α-treated tumors started to show onset of regression.

3.2. Changes in vascular volumes

In each tumor, the enhancement kinetics following the i.v. injection of Gd-DTPA were measured from the series of T_1-weighted dynamic contrast-enhanced images. Figure 2 shows the mean enhancement kinetics measured from the

Table 1

The percentage change in tumor size and vascular volume in the control and treated tumors

	Volumetric change		Vascular change
	day 4/day 0	day 7/day 4	day 4/day 0
Control	+ (73 ± 53)%	− (40 ± 26)%[a]	− (62 ± 9)%[a]
rIL1-α	+ (140 ± 30)%	+(159 ± 19)%[b]	+(41 ± 9)%[b]
rTGF-β	− (80 ± 3)%	−(90 ± 3)%	− (55 ± 8)%
rIFN-γ	− (95 ± 4)%	−(100 ± 0)%	NA

[a,b] Note that the vascular volume change of (day 4/day 0) predicts the volumetric change of (day 7/day 4). NA: not available due to tumor regression.

Table 2

The percentage change in tumor size and vascular volume in the control and treated tumors

	Volumetric change		Vascular change
	day 7/day 4	day 11/day 7	day 7/day 4
Control	− (40 ± 26)%	−(80 ± 3)%	NS
rIL1-α	+ (159 ± 19)%	+ (41 ± 16)%[a]	− (59 ± 17)%[a]
rTGF-β	− (90 ± 3)%	−(100 ± 0)%	NA
rIFN-γ	− (100 ± 0)%	NA	NA

[a] Note that the decrease of vascular volume of (day 7/day 4) correlates with the slow down of the tumor growth (from +159% at day 7/day 4 down to +41% at day 11/day 7).

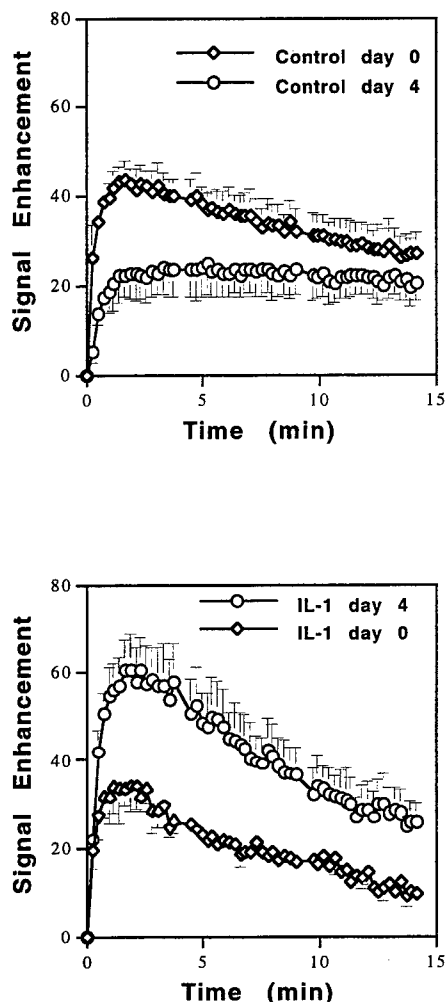NS: not significant (p > 0.05), NA: not available due to tumor regression.

Fig. 2. The enhancement kinetics of the control (a) and IL-1α treated (b) tumors, measured in the dynamic T_1-weighted images following administration of Gd-DTPA (0.1 mmol/kg) from the tail vein. Time 0 indicates the starting of injection, which took approximately 5 s to complete. The kinetics measured at day 0 (baseline) and day 4 (3 days post treatment) are shown on the same scale. Compared to the baseline, the enhancement level at day 4 decreased in the control tumors, also the decay pattern at day 0 has changed to a flat pattern at day 4, indicating decrease of vascularity. In contrast, the enhancement level increased in the IL1-α-treated tumor at day 4, and both curves demonstrated a similar pattern, indicating increase of vascularity.
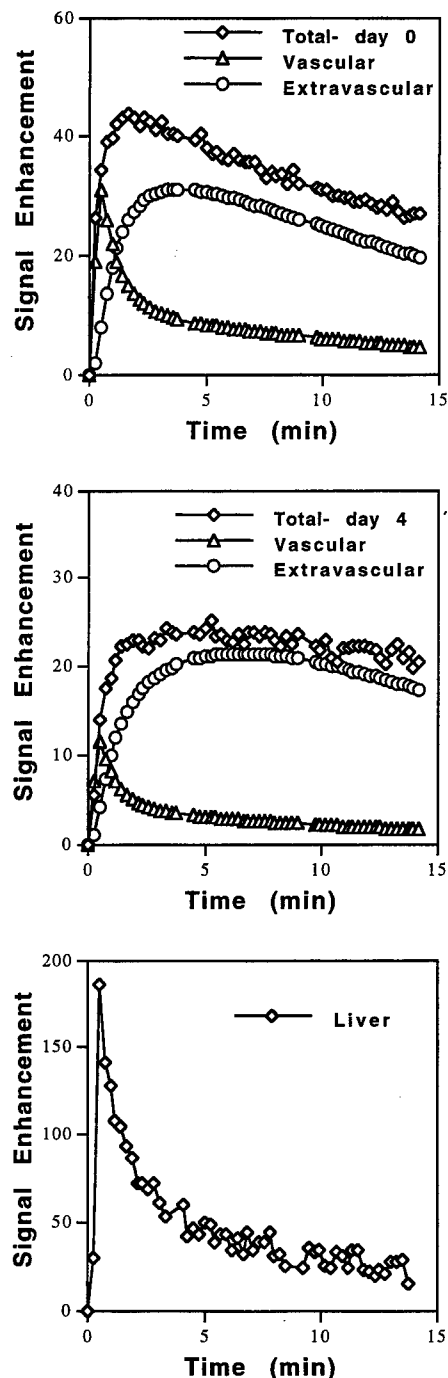
Fig. 3. The example of the pharmacokinetic analysis to separate the vascular vs. extravascular kinetics from the measured kinetics from the whole tumor. The kinetics from the control tumors, as shown in Fig. 2a, were analyzed. The total kinetics and the vascular and extravascular components at day 0 (a), day 4 (b), and the enhancement kinetics measured from the liver of the rat (c) are shown. By referencing to the liver kinetics (the distribution is in the entire extracellular space, assumed to be 24%), the vascular kinetics of the tumor was converted into vascular volume, which was about 4.2% at day 0 and 1.6% at day 4.

control tumors and the rIL-1α-treated tumors at day 0 and day 4. In control tumor, the enhancement level decreased significantly at day 4. In contrast, the enhancement level measured from the IL1-α-treated tumor increased after treatment. The changes are also significant. A two-compartmental pharmacokinetic model was applied to analyze the kinetics. The kinetics from the liver were also measured. The decay rate in the liver was used as the decay rate in the blood. By using the non-linear least squares fitting we can separate the vascular and extravascular components, as shown in an example given in Fig. 3. The mean kinetics of the control tumors shown in Fig. 2a were analyzed. Then by referencing to the liver kinetics (shown in Fig. 3c), the

vascular component was converted to fractional vascular volume. We assume the distribution volume is the total extracellular volume in the liver, 24%. Therefore, the vas-

cular volume as analyzed from the mean kinetics of the control tumor at day 0 is about 4.2%, and that at day 4 is decreased down to 1.6%. The analysis was performed for each tumor measured at each time point separately, and the percentage change of each individual tumor was calculated with respect to its own baseline value. The mean percentage changes in the control and IL1-α-treated tumors at day 4 compared to day 0 is shown in Table 1. The changes in rIL-1α treated tumors at day 7 compared to day 4 is shown in Table 2 (the vascular volumes in control tumors were measurable, but did not show a significant change among the four tumors). In Table 1, although the tumor size in both control and rIL-1α-infected animals increased at day 4, the vascular volume of control tumors decreased by 62% (compared to baseline), while the rIL-1α inoculated animal had an increase of 41%. These changes of vascular volume at day 4 were predictive of tumor growth at day 7. At day 7, control tumors had decreased by 40% while the rIL-1α infected tumors increased by 159%. In Table 2, it demonstrates that at day 7 the vascular volume of the rIL-1α animal tumors decreased by 59%. Again the change was predictive of future tumor growth. The growth rate of the rIL-1α-treated animal slowed down much (from 159% increase at day 7 down to 40% increase at day 11), and tumor started to regress by day 15.

4. Discussion

The C6 tumors we have established are allogeneic, which means that they will regress after the initial tumor is formed, due to the immune response against a different histocompatibility locus. However, treatment of these tumors with various recombinant adenoviruses can alter their usual growth and regression pattern. By using β-gal as a marker gene, we have shown that the C6 cells in culture and the established tumor in the animal did express the gene soon after they were infected with the same adenovirus system. In the present study our interest was to determine if these tumors and their treatment with various recombinant adenoviruses could provide a well-suited model for us to investigate the relationship between tumor growth and vascularity changes. Our interest, therefore, was not to study the mechanism of how the recombinant virus treatment works, but to evaluate if we can predict these different growth patterns with changes in vascularity measured by dynamic contrast-enhanced MRI.

In our bilateral tumor model, only the left side tumor was injected with the virus. However, the biological effects were seen bilaterally demonstrating that the effects of the recombinant adenovirus infection were systemic and not localized to the site of injection. This suggests that immunological processes are involved. The three genes used in this study, human transforming growth factor β, mouse interleukin 1α, mouse interferon γ are cytokines, which are low molecular weight proteins that regulate various aspects of the immune

response. They can assist in regulating both the development of immune effector cells and also possess direct effector [15]. However, the effects of cytokines can be very complicated and can vary in different biological situations. Therefore, a dynamic system to evaluate the ongoing consequence of recombinant cytokine gene therapy should be very valuable. The four control tumors from two different rats all showed the same growth patterns, and the bilateral tumors in each animal infected with each cytokine expressing virus also showed the same response. These findings suggested that infecting the left side tumor triggered the host immune system to respond, which consequently altered the growth of both tumors. Two months after the regression of tumors, these same five animals were injected with C6 tumor cells again, and none of them developed tumor. The results further support the view that the tumor rejection was due to immune response, and that the gene therapy used in this study altered the immune response for tumor rejection.

In this study, we demonstrated that the changes in vascular volume, determined using dynamic contrast-enhanced MRI, is predictive of future tumor growth. In all six tumors, which were increasing in tumor size, a decrease in tumor size was preceded by a decrease in vascular volume by several days. The predictive value was valid for both unmodulated allogeneic tumor rejection (four untreated tumors) and when there is modulation of an immune response (two tumors from the rIL-1α treated animal) resulting in a delayed onset of tumor rejection. Immune modulation that increased the immune response (rTGF-β and rIFN-γ) resulted in an accelerated tumor rejection. After the treatment the tumor size became too small for the vascularity measurements, thus the evaluation of vascular volume as a predictive tool could not be done in these two rats.

The enhancement kinetics of Gd-DTPA were analyzed by a pharmacokinetic model to obtain the vascular volume in the current study. Three major factors determine the kinetics of contrast agents in a tissue: blood perfusion, transport of agents across the vessel wall (via diffusion for the small size agent as Gd-DTPA), and diffusion of agents in the interstitium. Perfusion determines the amount of agents delivered to the tissue by blood, thus it is the first dominant factor determining the initial kinetics of the agent in the tissue. When the perfusion is sufficient, the transport of agent across the vessel wall, which is governed by permeability-surface area product (PS) of the vessel wall, is the second factor determining the early kinetics after injection of contrast agent. In our analysis the initial rise phase in the measured enhancement after contrast agent injection is attributed to vascular distribution. However, since the size of Gd-DTPA is relatively small, it can easily diffuse out of the vessel. Therefore, the vascular space volume obtained from the analysis was actually an "apparent vascular volume," which included the true vascular volume, and the fast leakage volume into the interstitial space [6]. Nevertheless, since the agents were delivered into the tumor by blood perfusion, and the percentage change in each individual

tumor was obtained from longitudinal studies, the measured vascular volume change is still a good indicator of the vascularity change (specifically include vascular volume and permeability) occurring in the tumor. We have previous shown that by using a macromolecular agent the vascular volume can be measured more accurately. However, none of the macromolecular agents suitable for tumor imaging have been approved by FDA (Food and Drug Administration) for clinical use yet.

We have applied the similar MRI measurement techniques to monitor the treatment efficacy in a mammary tumor model after chemotherapy [16]. In that study we used a blood pool agent (Gadomer-17) and a small agent (Gd-DTPA), and compared their ability for assessment of the vascularity change and their discriminative power for differentiating responders from controls. We found that although the blood pool agent was more sensitive to the vascularity change, and it could give differential changes earlier after the therapy, the small agent also gave significant vascular changes between responder and controls at a later time. Therefore, although none of the blood pool agents is mature for cancer imaging in humans yet, the dynamic contrast enhanced MRI with the clinically available small agent can still provide valuable information towards this goal. It may also serve as an important tool in understanding the gene therapy induced vascular changes. This technique can potentially be used to assess the efficacy of immunological and other gene therapy treatments at an early time, allowing for therapeutic modulation prior to the onset of tumor growth changes. Considering that gene therapy is still in its infancy stage, any additional information to aid in the understanding of the therapy induced changes will greatly help its future developments.

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